CARRIER:NUCLEIC ACIDS COMPLEXES CONTAINING NUCLEIC ACIDS ENCODING ANTI-ANGIOGENIC PEPTIDES AND THEIR USE IN GENE THERAPY

This application is a continuation in part of application Serial Number 985,526, filed December 5, 1997, which is a continuation in part of application Serial Number 680,845, filed July 16, 1996.

FIELD OF THE INVENTION

The present invention relates to delivery of antiangiogenic genes or nucleic acid encoding anti-angiogenic peptides to a tumor in vivo, and expression of the nucleic acid to inhibit tumoral growth. Carrier:nucleic acid complexes are provided comprising nucleic acid encoding at least one anti-angiogenic protein or peptide, optionally together with further nucleic acid encoding a tumor suppressor protein. These complexes are useful in gene therapy for inhibition of tumor growth.

BACKGROUND OF THE INVENTION

I. Gene Therapy

Development of gene therapy techniques is approaching clinical realization for the treatment of neoplastic and metabolic diseases. There remains substantial need for improvement both in the vector delivery systems for delivery of the transgene to target tissues, and the identification of genes most effective for anti-tumor therapy.

Vectors for carrying genes may be viral or non-viral. For example, replication-deficient retroviral vectors can efficiently transfect dividing cells. Local intratumoral injection of retroviruses that contain a thymidine kinase transgene has been used successfully to affect regression of gliomas (Culver et al, *Science*, 256:1550-1552 (1992)). Unlike retroviral vectors,

adenoviral vectors can also transfect non-dividing cells, and their ability to cause insertional mutagenesis is greatly reduced. However, they can have the undesirable potential to activate the immune system in humans (Crystal, *Science*, 270:404-410, (1995). Attempts are underway to minimize the immunogenicity of the adenoviral vectors.

Non-viral vectors of DNA include primarily liposomes, peptides, proteins and polymers (Ledley, *Current Opinion in Biotechnology*, 5:626-636 (1994)). Of these, liposomes are currently the most common non-viral vectors of DNA. The major advantage of liposomes over retroviruses is that DNA is not incorporated into the genome, and unlike adenoviral vectors, they are not immunogenic. However, the major limitation of liposomes is that they are not as efficient as viral vectors in transfecting many cell types. Until recently, their medical utility was limited by their rapid uptake by phagocytic cells. Interest in liposomes as a vector has been increased by two technological advances. First, stearically stabilized (Stealth) liposomes have been developed which are more non-reactive and are not readily taken up by the reticuloendothelial system (RES). Stealth liposomes are composed of lipids rich in oxygen in their head group (ethylene glycol or glycolipids) which provide a stearic barrier outside of the membrane. As a result, Stealth liposomes remain in the blood for up to 100 times longer than conventional liposomes, and can thus increase pharmacological efficacy (Papahajopoulos, *In*: *Stealth Liposomes*, Ed., Lasic et al, CRC Press (1995); and Lasic et al, *Science*, 267:1275-76 (1995)). However, stealth liposomes are still not particularly efficient in transfection of cells or as vectors for DNA.

The second significant advance in liposome technology has

been the use of cationic liposomes complexed to negatively-charged DNA. Cationic liposomes can condense DNA, and increase transfection yields several orders of magnitude. In the cationic liposome:DNA complex, the nucleic acids or oligonucleotides are not encapsulated, but are simply complexed with small unilamellar vesicles by electrostatic interactions. The exact nature of the cationic liposome:DNA complex is not fully known, but intricate topological rearrangements of the cationic liposome:DNA complex may occur, including DNA condensation, liposome aggregation, and fusion. This supramolecular complex can be added to cells *in vitro*, injected parenterally, or aerosolized for pulmonary applications (Lasic et al, *Science*, 267:1275-1276 (1995)). Further, the intravenous injection into mice of high concentrations of the CAT gene (100 μg or greater) complexed with cationic liposomes has been found to result in 40% transfection efficiency of well vascularized tissues, such as the spleen (Zhu et al, *Science*, 261:209-211 (1993)). Notwithstanding these advances, a major challenge of gene therapy remains the systemic delivery of transgenes to the tumor or peritumoral area that will effectively decrease the size of primary tumors and their metastases. Unlike the spleen and bone marrow, which are highly vascular and have a high capacity to filter macromolecules from the blood stream, most organs and tumors do not have this capacity, and the transfection efficiency of these tissues with liposomes is low (Marshall, *Science*, 269:1051-1055 (1995)). In addition, another limitation of cationic liposome: DNA complexes is that their ½ life in the blood stream is normally less than one

hour (Allen et al, In: *Liposome Technology*-Vol. III, Ed., Gregoriadis G et al, CRC Press (1993); Li and Huang, J. of Liposome Research, 6:589 (1996). Sufficient transfection of the target cell by vectors carrying therapeutic genes has thus far been the rate-limiting step in gene therapy. In addition to DNA, liposomes can bind and transport RNA into cells. In fact, the level of protein expression from transfected RNA is similar to the level of protein expressed from transfected DNA (Malone et al., *Proc. Natl. Acad. Sci. USA* 86:6077-6081 (1989).

II. Tumor Suppressor Genes

Tumor suppressor genes are well-known in the art, and include the p53 gene (Baker et al, *Science*, 249:912-915 (1990)), the p21 gene (El-Deiry et al, *Cell*, 75:817-825 (1993); and Harper et al, *Cell*, 75:805-816 (1993)), and the rb gene (Bookstein et al, *Science*, 247:712-715 (1990)).

Mutations in the tumor suppressor gene p53 are known to occur in over 50% of human tumors, including metastatic breast cancer. Various groups have found that reintroduction of the wild-type P53 by mediated transfer of a single copy of the p53 transgene into a variety of tumor cells, including breast cancer cells, results in a decrease in growth rate and/or attenuated tumor development once those transfected cells were implanted into nude mice (Wang et al, *Oncogene*, 8:279-288 (1993); Baker et al, *Science*, 249:912-915 (1990)); Bookstein et al, *Science*, 247:712-715 (1990); Cheng et al, *Cancer Res.*, 52:222-226 (1992); Isaacs et al, *Cancer Res.*, 51:4716-4720 (1991); Diller et al, *Mol. Cell. Biol.*, 10:5772-5781 (1990); Chen et al, *Oncogene*, 6:1799-1805 (1991); and Zou et al, *Science*, 263:526-529 (1994)). In addition, intratracheal injection of a retrovirus containing the p53 transgene has been

shown to significantly inhibit the growth of lung tumors (Fujiwara et al, *J. Natl. Cancer. Inst.*, 86:1458-1462 (1994)).

Systemic intravenous administration of a β-actin promoter-containing vector containing the p53 coding sequence complexed to cationic liposomes has been found to affect the tumor growth of a malignant line of breast cancer cells injected into nude mice (Lesoon-Wood et al, *Proc. Am. Ass. Cancer Res.*, 36:421 (1995); and Lesoon-Wood et al, *Human Gene Ther.*, 6:39-406 (1995)). Of the 15 tumors treated in this study, four of these tumors did not respond to treatment. Because of the unresponsiveness of these tumors, new therapies were sought in the present invention to more effectively decrease the size of these tumors.

p53 coordinates multiple responses to DNA damage. DNA damage results in an increase in the level of the p53 protein. Following DNA damage, an important function of wild-type p53 function is to control the progression of cells from G1 to S phase. Recently, several groups have found that p53 transcriptionally activates a p21 kd protein (also known as WAF1 or CIP1), an inhibitor of cyclin-dependent kinases (CDKs) (El-Deiry et al, *supra*; and Harper et al, *supra*). Inhibition of CDK activity is thought to block the release of the transcription factor E2F, and related transcription factors from the retinoblastoma protein RB, with consequent failure to activate transcription of genes required for S phase entry (Harper et al, *supra*; and Xiong et al, *Nature*, 366:701-704 (1993)). Evidence consistent with the model that pRb is a downstream effector of p53-induced G1 arrest has recently been reported (Dulic et al, *Cell*, 76:1013-1023 (1994)). Thus, p53 regulates cell cycle through two proteins: p21 and rb.

III. Anti-Angiogenic Proteins

Proteins with anti-angiogenic activities are well-known and include: thrombospondin I (Kosfeld et al, *J. Biol. Chem.*, 267:16230-16236 (1993); Tolsma et al, *J. Cell Biol.*, 122:497-511 (1993); and Dameron et al, *Science*, 265:1582-1584 (1995)), IL-12 (Voest et al, *J. Natl. Cancer Inst.*, 87:581-586 (1995)), protamine (Ingber et al, *Nature*, 348:555-557 (1990)), angiostatin (O'Reilly et al, *Cell*, 79:315-328 (1994)), laminin (Sakamoto et al, *Cancer Res.*, 5:903-906 (1991)), endostatin (O'Reilly et al., Cell, 88: 277-285 (1997)), and a prolactin fragment (Clapp et al, *Endocrinol.*, 133:1292-1299 (1993)). In addition, several anti-angiogenic peptides have been isolated from these proteins (Maione et al, *Science*, 247:77-79 (1990); Woltering et al, *J. Surg. Res.*, 50:245-251 (1991); and Eijan et al, *Mol. Biother.*, 3:38-40 (1991)).

Thrombospondin I (hereinafter "TSPI") is a large trimeric glycoprotein composed of three identical 180 kd subunits (Lahav et al, *Semin. Thromb. Hemostasis*, 13:352-360 (1987)) linked by disulfide bonds (Lawer et al, *J. Cell Biol.*, 103:1635-1648 (1986); and Lahav et al, *Eur. J. Biochem.*, 145:151-156 (1984)). The majority of anti-angiogenic activity is found in the central stalk region of this protein (Tolsma et al, *supra*). There are at least two different structural domains within this central stalk region that inhibit neovascularization (Tolsma et al, *supra*).

Besides TSPI, there are six other proteins (fibronectin, laminin, platelet factor-4, angiostatin, endostatin and prolactin fragment) in which peptides have been isolated that inhibit angiogenesis. In addition, the dominant negative fragment of FlK1 and analogues of the peptide somatostatin are known to inhibit angiogenesis.

Fibronectin (FN) is a major surface component of many normal cells, as well as a potent cell spreading factor. During transformation, the loss of cellular FN has been observed. Furthermore, the addition of fibronectin to transformed cells restores the normal phenotype. It has been found that either heparin-binding or cell-adhesion fragments from FN can inhibit experimental metastasis, suggesting that cell surface proteolyglycans are important in mediating the adhesion of metastatic tumor cells (McCarthy et al, *J. Natl. Cancer Inst.*, 80:108-116 (1988)). It has also been found that FN and one of its peptides inhibits *in vivo* angiogenesis (Eijan et al, *Mol. Biother.*, 3:38-40 (1991)).

Laminin is a major component of the basement membrane, and is known to have several biologically active sites that bind to endothelial and tumor cells. Laminin is a cruciform molecule that is composed of three chains, an A Chain and two B chains. Several sites in laminin have been identified as cell binding domains. These sites promote cellular activities *in vitro*, such as cell spreading, migration, and cell differentiation. Two peptides from two sites of the laminin B1 chain are known to inhibit angiogenesis (Grant et al, *Path. Res. Pract.*, 190:854-863 (1994)).

Platelet factor-4 (PF4) is a platelet α-granule protein originally characterized by its high affinity for heparin. The protein is released from platelets during aggregation as a high molecular weight complex of a tetramer of the PF4 polypeptide and chondroitin sulfate, which dissociates at high ionic strength. PF4 has several biological properties including immunosuppression, chemotactic activity for neutrophils and monocytes as well as for fibroblasts, inhibition of bone resorption, and inhibition of angiogenesis. The angiostatic properties of human PF4 are

associated with the carboxyl-terminal, heparin binding region of the molecule. A 12 amino acid synthetic peptide derived therefrom has been discovered to have marked angiostatic affects (Maione et al, *Science*, 247:77-79 (1990)).

Endostatin is a 20 kDa protein fragment of collagen XVIII. It has recently been found to be a potent inhibitor of tumor angiogenesis and tumor growth (O'Reilly et al., Cell, 88, 277-285, 1997).

Although somatostatin is not a protein, it is a naturally-occurring cyclic 14 amino acid peptide whose most-recognized function is the inhibition of growth hormone (GH) secretion. Somatostatin is widely distributed in the brain, in which it fulfills a neuromodulatory role, and in several organs of the gastrointestinal tract, where it can act as a paracrine factor or as a true circulating factor. The role played by the neuropeptide somatostatin, also known as somatotropin release inhibitory factor (SRIF), in human cancer is not well understood. Recent investigations involving somatostatin receptors in normal and neoplastic human tissues suggest that the action is complex, and involves both direct and indirect mechanisms. One of the anti-tumor mechanisms of these synthetic somatostatin analogues may be an anti-angiogenic effect (Woltering et al, *J. Surg. Res.*, 50:245-50 (1990)). In a recent study, the ability of native somatostatin and nine somatostatin analogues to inhibit angiogenesis were evaluated. The most potent somatostatin analogue was found to be approximately twice as potent as the naturally-occurring somatostatin (Barrie et al, *J. Surg. Res.*, 55:446-50 (1993)).

Angiostatin is a 38 kDa polypeptide fragment of plasminogen. Whereas plasminogen has no fibrinogenic activity, angiostatin has

marked angiogenic activity (O'Rielly MS, et al Cell, 79:315-28 (1994)). Angiostatin was isolated when it was observed that the primary tumor suppressed metastases. That is, when the primary tumor was removed, the metastases grew. Administration of angiostatin blocks neo-vascularization and growth of metastases.

The Flk1 receptor is a receptor for vascular endothelial growth factor (VEGF). FlK-1 is exclusively expressed on the surface of the endothelial cells. Once VEGF binds to the receptor, the Flk-1 receptor then homodimerizes to stimulate the endothelial cell to divide. If a mutant receptor of Flk-1 is transfected into the endothelial cells, the mutant receptor dimerizes with the wild-type Flk-1 receptor. In this endothelial transfected with the mutant Flk-1 receptor, VEGF is unable to stimulate the endothelial cells to divide. Co-administration of a retrovirus carrying the Flk-1 cDNA (Millauer B. et a., Nature, 367, 1994) inhibits tumor growth. This emphasizes that the receptor plays a critical role in the angiogenesis of solid tumors.

Finally, a 16kd fragment of prolactin has been found to be antiangiogenic. Similar to plasminogen, prolactin is not anti-angiogenic but the prolactin fragment is a potent in vivo and in vitro inhibitor of angiogenesis (Clapp C. et al. Endocrinology. 133:1292-1299 (1993).

Despite the evidence that anti-angiogenic peptides can be useful anti-tumor agents, and interest in targeting genes toward the vasculature, there have been no published reports on effective *in vivo* gene therapy regimens utilizing anti-angiogenic DNA sequences.

The only transfected antiangiogenic gene that has been shown to inhibit tumor growth is full length thrombospondin I. In that

study *(Weinstat-Saslow et al, Cancer Research 54, 6504-6511, (1994))* tumor cells that expressed 15-fold higher levels of the thrombospondin I in vitro than baseline cells were implanted into mice. This transfected full length thrombospondin I was secreted from the tumor cells, and effectively reduced the tumor by 60%. Thus, this study determined that transfection of 100% of the tumor cells with a highly expressed and secreted antiangiogenic protein was able to reduce tumor size.

SUMMARY OF THE INVENTION

An object of the invention is to deliver anti-angiogenic genes and/or nucleic acid encoding anti-angiogenic peptides to a tumor site in vivo, preferably by injection, whereby the nucleic acid is expressed to inhibit tumoral growth.

A further object of the present invention is to provide carrier complexes containing nucleic acid encoding anti-angiogenic peptides. The carrier may be specifically targeted to the tumor and/or to the tumor vasculature. The complexes are useful for providing anti-angiogenic gene therapy and inhibiting tumor growth in a subject.

A further object of the present invention is to provide carrier complexes containing nucleic acid encoding an anti-angiogenic gene or peptide, or DNA encoding more than one anti-angiogenic gene or peptide, and additionally nucleic acid encoding a tumor suppressor gene.

In currently preferred embodiments, the carrier material comprises complexes of cationic polymer or cationic liposomes and nucleic acid encoding one or more antiangiogenic peptides, optionally with nucleic acid encoding a tumor suppressor gene.

The complexes are administered in a tumor-inhibiting

effective amount to a patient, preferably by injection of the complexes.

DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph depicting the results of the experiment described in Example 8, infra, wherein complexes containing DNA encoding anti-angiogenic peptides were administered intratumorally.

Figure 2 is a graph showing the results of in vitro transfection experiments into endothelial cells using cationic polymer carrier complexed with DNA encoding anti-angiogenic peptides, as described in Example 10.

DETAILED DESCRIPTION OF THE INVENTION

In one embodiment, the above-described objects of the present invention have been met by a carrier:DNA complex comprising DNA encoding at least one anti-angiogenic gene or peptide and optionally additional nucleic acid encoding a tumor suppressor protein. The nucleic acid may encode a full-length anti-angiogenic protein, or may encode a peptide having antiangiogenic activity, or a combination of nucleic acids. The term "complex" refers to any hydrophobis and/or ionic interaction of nucleic acids with the viral or non-viral carriers. In addition, viral:nucleic acid complexes can also be defined as incorporation of the nucleic acid within the viral shell and/or insertion of the nucleic acid within the viral genome.

Preferred carrier vehicles are liposomes, polymers, viruses (retroviruses, adenoviruses, and adeno-associated viruses, for example), viral shells, micelles, microspheres and the like. See, e.g. Nabel, E., Vectors for Gene Therapy, in Current Protocols in

Human Genetics on CD-ROM, John Wiley and Sons (1997) The carrier used in the invention is selected such that it can deliver the DNA in vivo to a tumor and/or the peritumoral area, including tumor vasculature, in a manner such that the DNA can be expressed.

Liposome carriers are known in the art. Reference is made to, for example, Liposome Technology, 2d Edition, CRC Press: Boca Raton (1983); and Stealth Liposomes, Lasic and Martin, Eds., CRC Press: Boca Raton (1995). Examples of cationic lipids include 1,2-dioleolyl-sn-glycero-3-ethylphosphocholine (Avanti, Birmingham, AL), 1,2-dimyristoyl-sn-glycero-3-ethylphosphocholine (Avanti, Birmingham, AL), and (2,3-diol-eyloxy)propyl-N,N,N-trimethylammonium chloride (DOTMA) (Syntex Corp., Palo Alto, CA).

The cationic lipids may be used in a mixture with dioleoylphosphatidylethanolamine (DOPE) (Avanti, Bimingham, AL). In the cationic liposome embodiment, the amount of cationic lipid present in the mixture is generally in the range of from 100 to 40 mol%, preferably about 50 mol%. The amount of DOPE present in the mixture is generally in the range of from 0 to 60 mol%, preferably about 50 mol%.

The liposomes may contain lipid derivatives of polyethylene glycol (PEG), referred to herein as "pegylated lipids". Components useful in creating pegylated lipids include, for example, 1,2-diacyl-sn-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol) 2000]. If pegylated lipid components are present, they are generally included in amounts of 0 to 10 mol%, preferably 1 to 5 mol%.

Cationic liposomes are prepared in a manner similar to other liposomes, for example, the cationic lipids with/or without DOPE are dissolved in a solvent, e.g., chloroform. The lipids are then dried in a round bottom flask overnight on a rotary evaporator.

The resulting lipids are then hydrated with sterile water over a 1 hr period to form large multilamellar vesicle liposomes. To decrease the size of the liposomes, one may sonicate or pass the liposomes back and forth through a polycarbonate membrane. The DNA is then added to a solution containing the liposomes after their formation.

Cationic polymer carriers useful in the context of this invention include polyethyleneimime (available from Avanti Lipids), polylysine (available from Sigma), polyhistidine (Sigma), and Superfect (available from Qiagen) or co-polymers of these carriers. Use of cationic polymer carriers for gene delivery in vitro and in vivo has been described in the literature, for example, by Goldman et al., Nature BioTechnology, 15:462 (1997).

Stealth liposomes concentrate in solid tumors possibly due to their "leaky" vessels. Although stealth liposomes' uptake into cells is decreased due to the pegylation of their surface, this decrease is more than offset by their prolonged half-life in the circulation. Thus, pegylated liposomes are good carriers of DNA. Micelles are closely related to liposomes except they lack a bipolar membrane. They are made up of polar lipids, some of which can be the same cationic lipids utilized in liposomes. Similar to liposomes, micelles are known to transfect cells with plasmid DNA (Zhang YP. Et al., Pharmaceutical Res. 14: 190-6, 1997; Labat-Moleur F. et al., Gene Therapy 3: 1010-7, 1996).

As known in the art, there are potential problems with the intravenous injection of viral vectors. However, viruses can deliver transgenes by regional intra-arterial and/or intratumoral injections. Construction of viral vector carrying transgenes has been extensively described and they have been used successfully in gene therapy. (Nable, E. In "Current Protocols in Human Genetics

on CD-ROM", John Wiley & Sons, Inc. 1997).

Delivery of the complexes to a target in vivo can be enhanced by including a ligand in the complex having affinity for a specific cellular marker. Ligands include antibodies, cell surface markers, viral peptides, and the like, which act to home the complexes to tumor vasculature or endothelial cells associated with tumor vasculature, or to tumor cells themselves, if a secreted form of the antiangiogenic DNA is delivered. An antibody ligand may be an antibody or antibody fragment specific towards a tumor marker such as Her2, CEA, ferritin receptor, or a marker associated with tumor vasculature (integrins, tissue factor, or β-fibronectin isoform). Antibodies or other ligands may be coupled to carriers such as liposomes and viruses, as is known in the art. See, e.g., Neri et al., Nature BioTechnology, 15:1271 (1997); Kirpotin, D. et al., Biochemistry 36:66 (1997) Cheng, Human Gene Therapy, 7:275 (1996); Pasqualini et al., Nature Biotechnology, 15:542 (1997); and Park et al., Proc. Am. Ass. Canc. Res. 38:342 (1997); Mori and Haung supra; and Nabel, supra. Alternatively, psuedotyping of a retrovirus may be used to target a virus towards a particular cell. Marin et al., Mol. Med. Today, 3:396 (1997).

In a further embodiment, the complexes further include a tumor suppressor gene. Examples of such tumor suppressor genes include the p53 gene, the p21 gene (El-Deiry et al, *supra*; and Harper, *supra*), and the rb gene (Bookstein et al, *supra*). The p53 gene is the currently preferred tumor suppressor gene.

The particular anti-angiogenic protein or peptide encoded by the anti-angiogenic DNA is not critical to the present invention. Examples of suitable peptides include:

(i) a fragment of thrombospondin I (TSPf) having the amino

acid sequence shown in SEQUENCE ID NO: 1. This fragment is encoded by the DNA sequence (nucleotides 1013-1650 of the TSPI gene) shown in SEQUENCE ID NO: 2.

ii) a concatamer of TSPf having the amino acid sequence of SEQUENCE ID NO: 3, which is encoded by the DNA sequence shown in SEQUENCE ID NO: 4.

iii) laminin peptide having the amino acid sequence shown in SEQUENCE ID NO. 5, which is encoded by the DNA sequence shown in SEQUENCE ID NO. 6.

iv) a concatamer of the laminin sequence having the amino acid sequence shown in SEQUENCE ID NO: 7, which is encoded by the DNA sequence shown in SEQUENCE ID NO: 8.

v) a peptide from platelet factor-4 having the amino acid sequence shown in SEQUENCE ID NO: 9, which is encoded by the DNA sequence shown in SEQUENCE ID NO: 10.

vi) a concatamer of the platelet factor-4 peptide having the amino acid sequence shown in SEQUENCE ID NO: 11, which is encoded by the DNA sequence shown in SEQUENCE ID NO: 12.

vii) a somatostatin inhibitor peptide having the amino acid sequence shown in SEQUENCE ID NO: 13, which is encoded by the DNA sequence shown in SEQUENCE ID NO: 14.

viii) a concatamer of somatostatin inhibitor having the amino acid sequence shown in SEQUENCE ID NO: 15, which is encoded by the DNA sequence shown in SEQUENCE ID NO: 16.

ix) fibronectin inhibitor peptide having the amino acid sequence shown in SEQUENCE ID NO: 17, which is encoded by the DNA sequence shown in SEQUENCE ID NO: 18.

x) a concatamer of fibronectin inhibitor peptide having the amino acid sequence shown in SEQUENCE ID NO: 19, which is encoded by the DNA sequence shown in SEQUENCE ID NO. 20.

xi) angiostatin peptide having the amino acid sequence shown in SEQUENCE ID NO: 21, which is encoded by the DNA sequence shown in SEQUENCE ID NO: 22.

xii) a concatamer of angiostatin peptide having the amino acid sequence shown in SEQUENCE ID NO: 23, which is encoded by the DNA sequence shown in SEQUENCE ID NO: 24.

xiii) prolactin peptide having the amino acid sequence shown in SEQUENCE ID NO: 25, which is encoded by the DNA sequence shown in SEQUENCE ID NO: 26.

xiv) a concatamer of prolactin peptide having the amino acid sequence shown in SEQUENCE ID NO. 27, which is encoded by the DNA sequence shown in SEQUENCE ID NO: 28.

xv) a peptide of Flk-1-DN having the sequence shown in SEQUENCE ID NO: 34, which is encoded by the DNA shown in SEQUENCE ID NO. 35.

xvi) a peptide of endostatin having the sequence shown in SEQUENCE ID NO: 36, which is encoded by the DNA shown in SEQUENCE ID NO: 37.

The above sequences are exemplary and not limiting on the scope of the invention. Certain domains of these fragments are known to have antiangiogenic activity, as reported in the literature. As will be apparent, some of these sequences are concatameric. Use of concatamers can increase the anti-angiogenic dosage level without changing the amount of vector necessary for delivery. The concatamers can extend up to approximately 4400 bases in length (the coding region of a large protein), and the number of concatamers possible will depend on the number of bases of a single anti-angiogenic peptide-encoding unit. As seen in the above examples, the concatamer repeats can be separated by intervening sequences.

For fibronectin, the range of concatamers would be about 2 to about 66. Although the maximum number of anti-angiogenic units for the TSPf is about 6, one can increase this concatameric number by deleting sequence less material to anti-angiogenic effects, such as the sequence shown in SEQUENCE ID NO: 29, where the corresponding amino acid sequence is shown in SEQUENCE NO: 30. In a similar manner, the concatameric number of the platelet factor-4 peptide, somatostatin inhibitor, angiostatin, and prolactin can be modified and increased.

Since more than one anti-angiogenic pathway exists, concatamers consisting of two or more types of inhibitor could be more effective than homogenous concatamers. For example, heterogeneous concatamers of TSPI and the fibronectin inhibitors can be inserted into the same vector. An example of such a heterogenous concatamer encoding DNA is shown in SEQUENCE ID NO: 31. In such heterogenous concatamers, the peptide-encoding repeats of each sequence may be linked in blocks and/or randomly.

The heterogeneous concatamers need not be limited to only anti-angiogenic peptides. For example, the protein angiostatin or the large polypeptide fragment of prolactin can be modified with genes encoding anti-angiogenic peptides. Again, the concatameric number will vary depending on the number of nucleotide bases of the unit angiogenic inhibitor. In a concatamer of large and small anti-angiogenic inhibitors, the ratio of large to small inhibitors is 0.1 to 0.9, preferably 1:1.

A translational start signal Met is included in the peptides as well as a transcriptional stop codon (TAA).

The *Sal*I sites present in the above-sequences are a useful cloning tool for insertion of the DNA into a vector, for example BAP vector, which is known to be useful for expressing proteins

efficiently in *vivo* from the β-actin promoter (Ray et al, *Genes Dev.*, 5:2265-2273 (1991)). Other restriction sites can be incorporated into the DNA for cloning into other vectors, as those in the art will readily appreciate.

Other useful vectors for containing the DNA sequences include plasmids with a simian viral promoter, e.g., pZeoSV (Invitrogen); the CMV promoter, e.g., pcDNA3, pRc/CMV or pcDNA1 (Invitrogen); or the phosphoglycerate kinase (PGK) promoter (Abud et al., Developmental Genetics, 19:51 (1996). Plasmids with a CMV promoter may contain an intron 5' of the multiple cloning site (Zhu et al, *supra*). Plasmids containing the BGH terminator instead of the viral SV40 polyA terminator, e.g., pcDNA3, pRc/CMV, pRc/RSV (Norman et al, IBC's 5th Annual Meeting (1995); and Invitrogen vectors), can also be employed in the present invention so as to increase the expression of the tumor suppressor gene and the anti-angiogenic peptide(s) in targeted cells.

Expression of the DNA encoding the tumor suppressor protein and the DNA encoding the anti-angiogenic peptide can be achieved using a variety of promoters. For example, the promoter can be a generalized promoter, such as the β-actin promoter, a simian viral promoter, or the CMV promoter, or a tissue specific promoter, such as the α-fetal protein promoter which is specific for liver (Kaneko et al, *Cancer Res.*, 55:5283-5287 (1995), the tyrosinase promoter which is specific for melanoma cells (Hughes et al, *Cancer Res.*, 55:3339-3345 (1995); or the enolase promoter which is specific for neurons (Andersen et al, *Cell. Molec. Neurobiol.*, 13:503-515 (1993)).

The plasmid vector may contain multiple promotors to enhance expression efficiency. Moreover, a plasmid vector may include IRES sequence (internal ribosome entry site) between different DNA

coding sequences, allowing for the translation of more than one peptide from the same transcript. Coding sequences can be associated with secretory sequences in the vector to enhance expression levels. In another embodiment of the invention, the vector may comprise an extrachromosomal replicating vector. In a further embodiment, RNA carries the coding sequence of antiangiogenic genes. See, e.g. Calos, TIG 12:463 (1996). These and other techniques to optimize expression are known to those in the art.

The particular amount of nucleic acid included in the complexes of the present invention is not critical, the amount of total nucleic acid administered in the complexes generally being in the range of about 0.005 to 0.32 μg/pM of carrier, preferably 0.045 to 0.08 μg/pM of carrier.

The nucleic acid encoding a tumor suppressor gene is generally present in an amount of from 0.0025 to 0.16 μg/pM of carrier, preferably 0.028 to 0.04 μg/pM of carrier. The nucleic acid encoding an anti-angiogenic peptide is generally present in an amount from 0.0025 to 0.16 μg/pM of carrier, preferably 0.028 to 0.04 μg/pM of carrier.

The mole ratio of the nucleic acid encoding the tumor suppressor gene to the nucleic acid encoding the anti-angiogenic peptide is also variable. Generally, the mole ratio is between 1:5 to 5:1, preferably about 1 to 1.

The nucleic acid encoding the tumor suppressor gene and the anti-angiogenic peptide may be contained on the same vector or on separate vectors. Different nucleic acids encoding anti-angiogenic peptides may be provided on the same or different vectors within the complexes.

In another embodiment, the above-described objects of the

present invention have been met by a method for inhibiting tumor growth in a subject comprising administering to a tumor-bearing subject a carrier:nucleic acid complex comprising nucleic acid encoding an anti-angiogenic protein or peptide(s) with or without additional nucleic acid encoding a tumor suppressor gene.

It is possible to treat different types of tumors. Examples of tumors which can be treated in accordance with the present invention include solid tumors, e.g., lung, colon, brain, breast and melanoma tumors. All of these tumors are very dependent on blood supply to sustain their growth.

The particular mode of administering the carrier:DNA complex of the present invention depends on various factors, but preferred modes include intravenous, subcutaneous or intratumoral injection. Intravenous injection is the preferred administration mode for distribution of the complex to the developing blood vessels of the tumor.

The amount of the carrier:nucleic acid to be administered will vary depending upon the age, weight, sex of the subject, as well as the tumor volume and rate of tumor growth in the subject. Generally, the amount of nucleic acid to be administered will be about 1 to 60 μg, preferably about 5 to 16 μg.

The following examples are provided for illustrative purposes and should not be construed as limiting the scope of the invention.

MATERIALS

Production of DNA Vectors

A. TSPI Vector

The coding region of the TSPI gene is known (GB Accession

code-X14787). The TSPI gene was inserted into the *Xba*I site of BAP vector (Ray et al, *supra*), producing TSPI vector, in which expression of the TSPI gene is controlled by the β-actin promoter.

More specifically, TSPI cDNA and Bluescript plasmid (Promega) were digested with *Hind*I and *Xba*I, and then the TSPI cDNA was ligated into Bluescript. Next, Bluescript containing the TSP cDNA and BAP vector were digested with *Sal*I and *Bam*HI, and TSPI cDNA inserted in the *Xba*I site of BAP vector. The correct orientation of the TSPI gene in BAP vector was confirmed by DNA sequencing.

B. TSPf Vector

TSPf vector is a vector containing a DNA fragment of the TSPI gene which has the two anti-angiogenic domains (nucleotides 992-1650) (Tolsma et al, *supra*), and a start codon and a stop codon.

The DNA fragment was prepared by PCR using thrombospondin I cDNA as template, and 100 pmoles of each of the following primers 5'-TAGGTCTAGA**ATG**ACTGAAGAGAACAAAGAG-3' (SEQUENCE ID NO: 32) and 5'-ATGGTCTAGA**TTA**GAGACGACTACGTTTCTG-3' (SEQUENCE ID NO: 33) to amplify nucleotides 1013 to 1650 of the TSPI gene. Both primers contain *Xba*I sites (underlined), the first primer contains an ATG start codon (in bold), and the second primer contains a TTA stop codon (reverse orientation in bold).

The resulting 638 base pair fragment of the TSPI gene (hereinafter "TSPf") encodes peptides that are known to be angiogenic inhibitors (Tolsma et al, *supra*).

After amplification, the DNA fragment was purified, digested with *Xba*I, and the digested fragment inserted into the *Xba*I site of BAP vector such that the expression of the TSPf gene was controlled by the β-actin promoter (Ray et al, *supra*; and

Lesoon-Wood et al, *Human Gene Ther.*, 6:395-405 (1995)). The correct orientation of the fragment in BAP vector was verified by digestion with *Bam*HI, and confirmed by DNA sequencing.

C. p53 Vector

The coding sequence of the p53 gene was cut from plasmid p1SVhp53c62 (Zakut-Houri et al, *EMBO J.*, 4:1251-1255 (1985)) with *Xba*I, and inserted into the multiple cloning sites of pGEM3Z vector (Promega, Madison, WI). Digestion of the resulting vector with *Sal*I and *Bam*HI generated a 1900 bp fragment that was then inserted into the *Sal*I and *Bam*HI sites of BAP vector such that expression of the p53 gene was controlled by the β-actin promoter. The correct orientation of the p53 gene in BAP vector was confirmed by DNA sequencing.

D. Laminim peptide vector was prepared by annealing together the following two oligonucleotides:

5'-CTATCGTCGACATGTATATTGGTTCTCGTTAAGTCGACCTATC-3' (SEQUENCE ID NO: 38) and

5'-GATAGGTCGACTTAACGAGAACCAATATACATGTCGACGATAG-3' (SEQUENCE ID NO. 39),which contain an anti-angiogenic fragment from laminin, start and stop codons, and *Xba*I restrictions sites. The annealed oligonucleotides were then digested with *Xba*I, and inserted into the *Xba*I site of BAP vector. The plasmid was sequenced to verify correct orientation.

E. Angiostatin vector was prepared by amplifying the angiostatin coding sequence of plasminogen cDNA using the following primers:

5'-AGTATCTAGAATGAGTGTATCTGTCACAATG-3' (SEQUENCE ID NO: 40)

and

5'-GAATTCTAGATCACCTATGAGGGGTTTGCTC-3' (SEQUENCE ID NO: 41)

The resulting amplified fragment, which contained a genetically engineered ATG start site and a TAA stop codon, was digested with *XBAI*, purified, and inserted into the *XbaI* site of BAP vector. The plasmid was sequenced to verify correct orientation.

F. Laminin peptide concatamer vector was prepared by initially annealing the following two oligonucleotides: 5'-CTATCGTCGACATGTATATTGGTTCTCGTAAAAGATATATTGGTTCTCGTGGTAAAAGAGATATTGGTTCTCGTGGTAAAAGATAAGTOGACCTATC-3' (SEQUENCE ID NO: 42) and 5'-GATAGGTCGACTTAT-3' (SEQUENCE ID NO: 43). The former oligonucleotide contains an anti-angiogenic fragment from laminin repeated four times, start and stop codons, as well as *XbaI* restrictions sites. The annealed oligonucleotides were then extended with PFU (Stratagene), digested with *SalI*, and inserted into the *SalI* site of BAP vector. The plasmid was sequenced to verify correct orientation.

Preparation of Cationic Liposome:DNA Complexes

A DOTMA:DOPE liposome mixture is known to efficiently transfect endothelial cells *in vitro* (Tilkins et al, *Focus*, 16:117-119 (1994)). Accordingly, liposome:DNA complexes were prepared using DOTMA:DOPE, in a 1:1 ratio, essentially as described by Debs et al, *J. Biol. Chem.*, 265:10189-10192 (1990). Similar liposomes preparations can be prepared by mixing, at a 1:1 ratio, DOPE with other cationic lipids, such as, 1,2-dioleolyl-sn-glycero-3-ethylphophocholine, and 1,2-dimyristoyl-sn-glycero-3-ethylphophocholine.

More specifically, a mixture of 400 nmoles of the DOTMA and

DOPE were dried overnight on a rotary evaporator. Then, the lipids were rehydrated with 1.5 ml of water for 2 hrs. Next, the milky liposome preparation was sonicated with a bath sonicator until clear. The resulting liposome preparation was then passed through a 50 nm polycarbonate filter between 15 to 20 times with a LipsoFast-Basic extruder (Avestin, Ottawa, On).

The DNA (see following examples) was prepared with the maxi Qiagen kits (Qiagen Inc., Chatsworth, Ca), and washed twice in 70% (v/v) ethanol. The DNA was then washed with distilled water or dialyzed against water for 24 hrs to removed any remaining salt.

About 400 pmols of the liposome preparation was gently mixed with between 10 to 35 μg of total DNA in an Eppendorf tube. This amount in each eppendorf tube was sufficient for two injections. The same amount of DNA was injected in the combination therapies as in the single treatment regimens. For example, if 16 μg of DNA in the combination therapy (8.0 μg of p53 + 8.0 μg of TSPf) was injected into each mouse of one group, then 16 μg of p53 was injected into each mouse of a second group.

EXAMPLE 1

The anti-angiogenic effects of carrier:DNA complexes were evaluated in mice containing MDA-MB-435 breast cancer tumors (American Type Tissue Culture, Bethesda, MD), which are p53 deficient.

More specifically, after administering the anesthetic, Metofane, to 126 female athymic nude mice(NCI), the mice were injected with 2.0 x 10^5 MDA-MB-435 tumor cells into the mammary fat pad using a stepper (Tridak) and a 27.5 g needle. Two weeks

later, the mice whose tumors grew were divided into various treatment regimens, 18 mice per each regimen. The treatment regimens were as follows: (1) untreated; (2) empty BAP vector; (3) TSPI vector alone; (4) TSPf vector alone; (5) p53 vector alone; (6) p53 vector + TSPI vector; and (7) p53 vector + TSPf vector. The mice received two intravenous injections, the first injection 14 days after the malignant cells had been implanted into the mice, and the second injection 24 days after the malignant cells had been implanted into the mice. The first injection consisted of 200 pmoles of the liposomes complexed with 16 μg of total DNA. The second injection consisted of 200 pmols of the liposomes complexed with 12.0 μg of total DNA. The sizes of the tumors were measured 7 days after the second injection. The results are shown in Table 1 below.

TABLE 1

Anti-tumor Effects of TSPI and TSPf

Putative Anti-tumor DNA	Tumor Size (mm^3)
Untreated	113.5±6.41
BAP	102.9±6.83
TSPI	103.2±8.96
TSPf	89.4±11.06
p53	80.1±12.7*
p53 + TSPI	82.9±6.95*
p53 + TSPf	53.2±8.37**

* p53 or p53 + TSPI vs. untreated, $p<0.05$

** p53 + TSPf vs. untreated or BAP, $p<0.01$

As shown in Table 1 above, the p53-treated group was found to

be statistically different from the untreated group ($p<0.05$) after 2 injections. However, the p53 treated group was not statistically different from the empty BAP vector group. This was similar to the results described by Lesoon-Wood et al, *Human Gene Ther.*, 6:395-406 (1995), in which p53 was not statistically different from the empty BAP vector group until after 5 injections.

However, p53 in combination with TSPf reduced tumors more effectively than p53 alone. After just 2 injections of this combination therapy, there was a 35% further reduction in tumor growth compared to p53 alone. The combination group was statistically different from both the untreated and the empty BAP vector groups ($p<0.01$). Although TSPf by itself was slightly less effective than p53, TSPf was, unexpectedly, substantially more effective than TSPI. In fact, the full length TSPI-treatment group had no more effect than either the empty vector or the untreated groups. This was unexpected for several reasons: 1) both the full length and the fragment of thrombospondin I contained the anti-angiogenic peptide, and 2) in a previous ex vivo study (Weinstat-Saslow et al, supra) full length thrombospondin I was effective in inhibiting tumor growth.

EXAMPLE 2

A second experiment was carried out to determine whether the combination therapy of p53 and TSPf was effective at lower dosages, and to confirm that the combination of p53 and TSPf reduced the tumor size significantly more than p53 alone.

More specifically, 36 mice were injected with 2.0×10^5 MDA-MB-435 tumor cells into the mammary fat pad. Two weeks later, the mice whose tumors grew were divided into various treatment

regimens, 12 mice per each regimen. The treatment regimens were as follows: (1) empty BAP vector; (2) p53 vector alone, and (3) p53 vector + TSPf vector. The mice were injected intravenously with 200 pmols of the liposomes complexed with 8.0 μg of total DNA. Subsequently, the mice were treated in the same manner with 200 pmols of the liposomes complexed with 12 μg of total DNA for the next 4 injections. Ten days elapsed between each injection. The sizes of the tumors were measured before each injection and 7 days after the last injection. The results are shown in Table 2 below:

TABLE 2

Anti-tumor Effects of p53 and TSPf

Putative Anti-tumor DNA	Tumor Size (mm^3)
BAP	855±345
p53	616±142
p53 + TSPf	265±133*

* p53 + TSPf vs. BAP, $p<0.02$

As shown in Table 2 above, the combination therapy with p53 and TSPf was statistically different from BAP, whereas the p53 alone treatment was not. This experiment confirmed that p53 and TSPf can be more effective than p53 alone. Furthermore, a different dosage regimen, without an initial booster dose of 16 μg of DNA as used in the experiment in Table 1, accentuated the difference between the combination treatment and the p53 alone treatments.

EXAMPLE 3

The experiment of Example 2 was repeated to confirm that BAP-TSPf complexed to liposomes effectively inhibited the growth of implanted tumors. Five injections of the liposome:DNA complex was administered intravenously to three groups: 1) BAP, 2) TSPF, or 3) p53. Results are shown in Table 3.

TABLE 3

Antitumor Effects of TSPf

Putative Anti-tumor genes	Tumor Size (mm^3)
BAP	619±65
TSPf	386±35*
P53	419±26*

*TSPF vs. BAP $p<0.05$

p53 vs. BAP, $p<0.05$

After five intravenous injections at a dose of 14.5 μg, the TSPf treatment gorup was statistically different form the BAP group.

EXAMPLE 4

An experiment was carried out to investigate the efficacy of complexes carrying DNA encoding anti-angiogenic peptide fragments of angiostatin and laminin.

126 mice injected with MDA-MB- 435 tumor cells as described in Example 2 were treated as follows: (1) BAP vector; (2) TSPf vector alone; (3) laminin peptide vector alone; and (4)

angiostatin vector alone. The mice received 4 intravenous injections, the first injection being 10 days after the malignant cells had been implanted into the mice, and the remaining injections were thereafter 10 days apart. The injections consisted of 200 pmols of the liposomes complexed with 12.5 μg of total DNA.

The results are shown in Table 4 below.

TABLE 4

Putative anti-tumor DNA	Tumor Size (mm^3)
BAP	194.7 ± 11.9
TSPf	135.9 ± 11.9*
Laminin peptide	126.4 ± 8.4*
Angiostatin	95.2 + 6.3*,**

* TSP., Laminin peptide, and Angiostatin vs. BAP, $p<0.05$

** Angiostatin vs. BAP, $p<0.01$

As shown in Table 4 above, the cationic liposomes containing DNA encoding anti-angiogenic peptides (TSPf, laminin peptide and angiostatin) significantly inhibited tumor growth.

EXAMPLE 5

MCF7 cells (American Type Tissue Culture, Bethesda, MD), which are a breast cancer cell line with two normal p53 alleles, were evaluated as described above except that 4.0×10^6 cells were injected into the mice and the third injection contained 12 μg of the DNA. Each injection was 10 days apart. Nine mice were injected with each of the following treatments except for regimen (1), in which 8 mice were treated: (1) untreated; (2) BAP; (3) p53; and (4) p53 + TSP. The sizes of the tumors were measured 7 days after the third injection. The results are

shown in Table 5 below.

TABLE 5

Effect of p53 and TSP. on MCF7s Cells

Putative Anti-tumor Genes	Tumor Size (mm^3)
Untreated	124.6±7.3
BAP	136±16.8
p53	83.1±13.6*
p53 + TSPf	69.0±13**

* p53 vs. untreated or BAP, $p<0.05$

** p53 + TSPf vs. untreated or BAP, $p<0.01$

As shown in Table 5 above, the most effective therapy against MCF7 was p53 and TSPI. The significance level for the p53 + TSPf therapy was greater than for p53 alone when they were compared against either the untreated or the BAP groups. The above experiment confirmed that p53 and TSPfI can decrease the MCF7 tumor more than the p53 treated or the untreated groups.

EXAMPLE 6

4 X 10^5 MCF7 cells were injected bilaterally into the mammary fat pads of the 28 nude mice. After two weeks of growth, these mice were randomly divided into four groups: 1) empty vector, 2) p53, 3) p53 + TSPf , and 4) untreated. The mice received one injection of 200 pmoles of liposomes complexed with 14 ugs of DNA, and the tumors from the various treatment groups were measured 10 days after the treatment. The results are shown in Table 6 below.

TABLE 6

Putative Anti-tumor Genes	Tumor Size (mm^3)
Empty vector-	54.7±4.0
p53	45.5±5.0
p53 + TSPf	33.9±3.6*
Untreated	61.9+8.3

*, p53 + TSPf vs Untreated, p<.025

As shown in Table 6 and previous tables, the additional reduction of the tumor by the combined use of p53 and TSPf compared to the use of p53 only, suggests that TSPf and p53 have different mechanisms of action. Although this does not preclude that the target of p53 is the vasculature of the tumor, the mechanism of inhibition of the tumor by p53 is uncertain at present. However, any mechanism of tumor inhibition by p53 and/or thrombospondin I must account for the low transfection efficiency of the tumor. Using a liposome complexed to a chloramphenicol acetyltransferase marker, it has been demonstrated that less than 5% of the tumor derived from MDA-MB-435 cells was transfected with the marker gene, and assuming similar transfection efficiency here, these favorable results were observed notwithstanding the very low level of transfection.

EXAMPLE 7

In a further experiment, it was determined that liposomes complexed to DNA encoding the laminin peptide can inhibit tumor growth. More specifically, after administering the anesthetic, Metofane, to 24 female athymic nude mice, the mice were injected with 3.0 x 10^5 MDA-MB-435 tumor cells into the mammary fat pad using a stepper and a 27.5 g needle. Two weeks later, the mice

whose tumors grew were divided into various treatment regimens, 8 mice per each regimen. The treatment regimens were as follows: (1) BAP, (2) laminin, and (3) p53 + laminin. The mice were injected intravenously with 200 pmols of the liposomes complexed with 12.5 μgs of total DNA 6.25 μg of each vector when a combination was used. the mice then received 3 injections, each 10 days apart. The tumors were measured at the time of each injection and at the time of the last injection. The results are shown in Table 7 below.

TABLE 7

Putative Anti-tumor Genes	Tumor Size (mm^3)
BAP	345 ± 23.5
Laminin peptide	280 ± 32.4
Laminin peptide + p53	192 ± 10.5*

*BAP vs. Laminin peptide + p53, $p<0.05$

As shown in Table 7 above, cationic liposomes containing a combination of DNAs encoding laminin peptide + p53 were more effective in reducing tumor growth than when DNA encoding the anti-angiogenic peptide was used alone. Thus, the addition of a tumor suppressor gene, p53, can enhance the anti-tumor effect of the anti-angiogenic peptide.

EXAMPLE 8

Although intravenous injection is preferred, the method of administration of the liposome:DNA complex is not critical. It has been found that intratumoral injections are effective. In an experiment involving intratumoral injection, 18 mice were injected with $3X10^5$ C6 glioma cells (rat brain tumors) subcutaneously. Six days after the injections, the mice were separated into 3 groups:

1) BAP, 2)FLK-DN (a dominant negative receptor), and 3) angiostatin. After the second intratumoral injection, there was a statistical difference between the angiostatin and the BAP groups. See Figure 1. Thus, the therapy of the invention is effective when complexes are administered intratumorally. The therapy is effective against tumors other than breast tumors.

EXAMPLE 9

It was also found that a liposome: secretory angiostatin construct can be more effective than the non-secreted analog. In this experiment, 24 nude mice were injected with $3X10^5$ MDA-MB-435 cells. Two weeks later the mice were divided into three groups, and received the following therapies intravenously:1) liposome:BAP, 2)liposome:secreted angiostatin, and 3)liposome:angiostatin. The concentration of DNA injected into the mice was 14.5 ugs. The mice received one injection of the liposome:DNA complex and their tumors were measured 10 days after the injection.

Table 8

Efficacy of Secretory Angiostatin

Therapeutic DNA	Tumor Size (mm^3)
Angiostatin	28.8±2.2
Angiostatin-Secretory	18.6±1.8*
BAP	30.5±3.3

*,P<0.05, BAP vs. Angiostatin-secretory

As seen in table 8, the secretory angiostatin treatment group was more effective than the vector control or the angiostatin treatment group in reducing the size of the tumor. From this experiment, it is demonstrated that a secretory sequence inserted

into the 5' portion of the antiangiogenic inhibitor can increase its efficacy.

EXAMPLE 10

Further experiments indicate that cationic polymers can be useful as carriers in the present therapy, and can be the carriers of choice under certain conditions.

In the following example, a cationic polymer (Superfect) was compared to cationic liposomes as carrier for transfecting endothelial cells in vitro with the CAT marker. The cationic liposomes used for comparison to the polymer were DOSPER (Boerhinger), which of 14 lipids screened in vitro gave the best results. In this experiment, $1X10^6$ Huvec cells were placed into each well of a 6 well plate. 25 uls of Superfect complexed with 2 ugs of DNA was added to each plate 24 hours after the initial seeding of the cells, and compared to plates to which had been added 2 ugs of DNA complexed with cationic liposomes. 36 hours after the transfection, the cells were lysed and the amount of CAT protein was assayed. The results are shown in Table 9.

Table 9

Vectors	Activity(DPMs/protein)
Cationic liposomes with BAP	31.1±7.2
Cationic liposomes with CAT	682±129
Superfect with BAP	21.4±0.458
Superfect with CAT	10816+687*

p<0.001, Superfect-CAT vs. Cationic liposome-CAT

This experiment suggests that a cationic polymer are superior in the transfection of endothelial cells, which is significant since we have hypothesized that endothelial cells of the tumor are a primary target of the therapeutic gene. Similarly, it has been found in some cell lines that Superfect is a better transfection agent in vitro than cationic liposomes.

EXAMPLE 11

Since Superfect appeared to be superior to cationic liposomes in the transfection of endothelial cells in vitro, it was investigated whether Superfect complexed to a therapeutic gene would inhibit tumor growth compared to the corresponding liposome complex. This experiment was based on the hypothesis that the endothelial cells and not other cells are the primary target of these cationic vehicle:DNA complexes. In this experiment, six mice were injected with 2.5 x 10^5 MDA cells into the mammary fat pad bilaterally. These tumors were allowed to grow to a large size for 2 months. At this size, the tumor growth is rapidly increasing at an exponential rate and is more resistant to treatment compared to smaller tumors. The mice were treated intravenously via the the tail vein with either the cationic liposome:BAP-p53/CMV-TSPf or Superfect:BAP-p53/CMV-TSPf. 9.5 µgs of DNA were complexed to Supefect (108 µg) or the cationic liposome (200 pmoles). The mice received only one dose of these therapies and their tumors were measured 10 days later. The mice tolerated both therapies without any apparent toxicity. The results are given in the table below.

TABLE 10

	Liposome	Superfect
Before Treatment	380±95#	384±86
After Treatment	525+80	403+72

#- tumor size in mm^3

The Superfect carrier appears to be superior to the liposome carrier even after one dose in these large tumors. There was only a minimal increase (5%) in the Superfect-treated group whereas there was a marked increase in the liposome-treated group (38%). When the growth of individual tumors were examined and compared to pre-treatment measurements, all 6 tumors in the liposome-treated group increased in their size. In contrast,4 of the 6 tumors in the Superfect group showed regression in their size compared to pre-treatment measurements.

EXAMPLE 12

This experiment was carried out using concatamer DNA encoding anti-angiogenic peptides. Mice injected with MDA-MB-435 tumor cells were treated as follows:

(1) BAP vector; (2) laminin peptide concatamer alone; and (3) laminin peptide vector alone. The mice received 2 intravenous injections, the first injection being 10 days after the malignant cells had been implanted into the mice, and the second injection 10 days later. The injections consisted of 200 pmols of liposomes complexed with 12.5 μg of vector DNA. The results are shown in Table 11 below.

TABLE 11

Putative Anti-tumor DNA	Tumor Size (mm^3)
BAP	86.8 ± 12.0
Laminin peptide concatamer	63.9 ± 4.8
Laminin peptide	53.7 + 3.0*

* Laminin peptide v. BAP, $p<0.05$

As shown in Table 11, complexes containing DNA encoding laminin concatamer or laminin peptide reduced tumor growth compared to the control (BAP vector).

EXAMPLE 13

To assess efficacy using a combination of DNAs encoding antiangiogenic peptides, mice injected with MDA-MB-435 tumor cells were treated as follows:

(1) BAP vector; (2) TSPf vector + angiostatin vector; (3) laminin peptide vector + TSPf vector; (4) laminin peptide vector + angiostatin vector; and (5) laminin peptide and FlK-DN receptor. The mice received 5 intravenous injections, the first injection being 10 days after the malignant cells had been implanted into the mice, and the remaining injections 10 days apart. The injections consisted of 200 pmols of liposomes complexed with 12.5 μg of total vector DNA, with 6.25 μg of each vector when a combination was used. The results are shown in Table 12 below.

TABLE 12

Putative anti-tumor DNA	Tumor Size (mm^3)
BAP	626 ± 78
TSPf + Angiostatin	296 ± 40*
Laminin peptide + TSPf	461 ± 54
Laminin peptide + Angiostatin	483 ± 46

Laminin Peptide and FlK-DN	482 ± 21

* TSPf + Angiostatin vs. BAP, $p<0.01$

As shown in Table 12, cationic liposomes containing combinations of DNA encoding anti-angiogenic peptides showed favorable inhibition of tumor growth.

While the invention has been described in detail and by reference to specific embodiments thereof, it will be apparent to one of ordinary skill in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof. Documents cited herein are incorporated by reference to the extent relevant to practicing the invention.